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Isolation and Characterization of a Bovine Trophectoderm Cell Line Derived From a Parthenogenetic Blastocyst

NEIL C. TALBOT,[1]* THOMAS J. CAPERNA,[2] ANNE M. POWELL,[1] WESLEY M. GARRETT,[1] AND ALAN D. EALY[3]

[1]*USDA, ARS, Animal and Natural Resources Institute, Biotechnology and Germplasm Laboratory, Beltsville Agricultural Research Center, Beltsville, Maryland*
[2]*Growth Biology Laboratory, Beltsville Agricultural Research Center, Beltsville, Maryland*
[3]*Department of Dairy and Animal Science, The Pennsylvania State University, University Park, Pennsylvania*

ABSTRACT A bovine trophectoderm cell line was established from a parthenogenetic in vitro-produced blastocyst. To initiate the cell line, 8-day parthenogenetic blastocysts were attached to a feeder layer of STO fibroblasts and primary outgrowths occurred that consisted of trophectoderm, endoderm, and very occasionally epiblast tissue. Any endoderm and epiblast outgrowths were removed from the primary cultures within the first 10 days of culture by dissection. One of the primary trophectoderm cell cultures was chosen for further propagation and was passaged by physical dissociation and replating on STO feeder cells. The cell culture, designated BPT-1, was maintained in T25 flasks and passaged at a 1:3 split ratio for the first 15 passages approximately once every 2 weeks. Thereafter, the cell culture was passaged at 1:10–1:40 split ratios. Transmission electron microscopic examination showed the cells to be a polarized epithelium with apical microvilli, a thin basal lamina, and lateral junctions consisting of tight junctions and desmosomes. Lipid vacuoles and digestive vacuoles were also prominent features of the BPT-1 cells. Metaphase spread analysis at passage 59 indicated a near diploid cell population ($2n = 60$) with a mode and median of 60 and a mean of 64. BPT-1 cells secreted interferon-tau into the medium as measured by anti-viral assay and Western blot analysis. The cell line provides an in vitro model of parthenogenote trophectoderm whose biological characteristics can be compared to trophectoderm cell lines derived from bovine embryos produced by normal fertilization or nuclear transfer. *Mol. Reprod. Dev. 69: 164–173, 2004.* © 2004 Wiley-Liss, Inc.

Key Words: bovine; cell; line; parthenogenetic; trophectoderm

INTRODUCTION

The trophoblast or trophectoderm is the first differentiated tissue (epithelium) of the early mammalian embryo (Betteridge and Fléchon, 1988). Although the trophectoderm does not contribute to the embryo itself, i.e., is an extraembryonic tissue, it plays varied and vitally important roles in preimplantation and post-implantation development. In the bovine embryo, it is morphologically defined by 6–7 days post-fertilization just after the morula stage and concurrent with the formation of the blastocyst (Betteridge and Fléchon, 1988; Plante and King, 1994). The trophectoderm comprises the outer most cell layer of the early embryo and transports fluid into the interior of the embryo to produce the typical fluid filled hollow ball formation that is the blastocyst (Massip et al., 1981; Plante and King, 1994). In the bovine embryo, the trophectoderm tissue undergoes a period of remarkable growth during the third week post-fertilization, and with this tissue expansion, the blastocyst changes to the narrow elongated tube form that typifies its peri-implantation morphology (Chang, 1952; Betteridge and Fléchon, 1988). During this time, the trophectoderm cells secrete interferon-tau (IFN-τ) which acts as the primary 'recognition of pregnancy' signal to the cow (Helmer et al., 1987). Gastrulation also begins at this time, and mesodermal cells spreading laterally from the embryonic disc begin to line the inner aspect of the trophectoderm thereby forming the chorion (Carlson, 1981; Betteridge and Fléchon, 1988). The allantosis develops from the third week on within the coelom of the expanded blastocyst (Betteridge and Fléchon, 1988). It is not until after the 5th week of gestation that the trophectoderm tissue, now in association with the allantosis tissue layer (chorioallantoic membrane), establishes its intimate association with the uterus to form the placental structures and begin acquisition of nutrients from the uterine endometrium directly (Chang, 1952; Mossman, 1987). Thus, the many

*Correspondence to: Neil C. Talbot, USDA, ARS, ANRI, BGL; Bldg. 200, Rm. 13, BARC-East; Beltsville, MD 20705.
E-mail: Ntalbot@anri.barc.usda.gov.

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important roles played by this extraembryonic tissue make it centrally important to the study of early embryo survival and the establishment of pregnancy.

In vitro cell culture models of bovine trophectoderm may be useful in the study of bovine early embryonic development and placentation. Of central interest to this laboratory is whether they may help illuminate differences between embryos created by somatic cell nuclear transfer (NT) and embryos derived from normal fertilization with egg and sperm. This is particularly relevant since failures in placentation appear to be a critical problem with NT pregnancy establishment and maintenance (Hill et al., 2000; De Sousa et al., 2001; Hashizume et al., 2002). Recently, in vitro cell culture models of bovine trophectoderm have been established (Talbot et al., 2000a; Shimada et al., 2001). Morphological features of the trophectoderm cells continuously cultured in vitro were similar to those found in vivo (Talbot et al., 2000a), and the cells also displayed functional characteristics of the in vivo elongated late stage bovine blastocyst such as IFN-τ and placental lactogen secretion (Talbot et al., 2000a; Nakano et al., 2002).

For the comparative study of possible mechanisms of placentation failure in NT pregnancies, an in vitro model of trophectoderm derived from a source of bovine embryos that never successfully implant, and that have deficiencies in their extraembryonic membrane form and function, would be useful. Trophectoderm cell lines derived from parthenogenetic bovine embryos might provide such a comparative model. In the mouse, parthenogenetic embryos are characterized by poor development of their extraembryonic membranes, and they uniformly fail to establish pregnancy (Surani and Barton, 1983; Surani et al., 1986). Much of the developmental failure in parthenogenotes is thought to arise from their lack of paternally imprinted genes (Surani et al., 1990). Likewise, the aberrant gene expression that occurs after cloning, some in imprinted genes, may be one of the causes of the high incidence of pregnancy failure in NT embryos (Humpherys et al., 2002; Inoue et al., 2002). While mammalian parthenogenote-derived cell lines are rare, they have been established from mouse embryos either in the form of embryonic stem (ES) cell lines (Kaufman et al., 1983) or embryonic fibroblast secondary cell cultures (Kharroubi et al., 2001).

The isolation of a trophectoderm cell line from a parthenogenetic 8-day bovine blastocyst is described. The continuous culture and characterization of the cell line were undertaken to demonstrate their basic similarity to, or differences from, another trophectoderm cell line previously isolated from a bovine blastocyst produced by in vitro fertilization (IVF).

MATERIALS AND METHODS

Cell Culture

All cells were grown on tissue culture plastic ware (Nunc, Denmark; and Falcon, Becton/Dickinson, Lincoln Park, NJ). Cryovials (2 ml) were purchased from Nunc. Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Cell culture reagents including, Dulbecco's phosphate buffered saline (PBS) without Ca^{++} and Mg^{++}, media, trypsin–EDTA (0.05% trypsin, 0.43 mM EDTA), antibiotics, nonessential amino acids, and L-glutamine were purchased from InVitrogen Corporation (GIBCO) (Gaithersburg, MD). STO cells (CRL 1503, American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagles medium with high glucose supplemented with 10% FBS (10% DMEM). Feeder layers were prepared by exposing a suspension of STO cells to 8 krad gamma radiation and plating the cells at 6×10^4 cells/cm^2. Feeder layers were maintained by refeeding with 10% DMEM every 6–7 days.

A primary bovine parthenogenetic trophectoderm (BPT-1) culture was initiated from a 8-day blastocyst stage parthenogenetic embryo, created as described previously (Talbot et al., 2000b), by pressing it onto the STO feeder cell monolayer and plastic with a hypodermic needle. No epiblast contamination was observed in the colony outgrowth that occurred over the first 2 weeks of primary culture, but a small amount of endoderm contamination was observed. The contaminating endoderm cells were removed in toto by dissection as described previously (Talbot et al., 2000a). After 3 weeks of primary culture, the colony, then approximately 1 cm in diameter, was passaged for secondary culture by physical dissociation as described previously (Talbot et al., 2000a). BPT-1 cells were cultured in 10% DMEM/199 medium (Talbot et al., 2000a) until passage 32. Subsequent passages were cultured in 10% DMEM.

BPT-1 Cell Growth Assays

Growth of the BPT-1 cells was assayed macroscopically by photo documentation of sister flasks that were simultaneously fixed and stained at progressively longer time points post-passage. Fixation and staining of the cultures were performed by aspirating the medium from the flasks and immediately replacing it with a fixing/staining solution of 0.125% Coomassie Blue R-250 (InVitrogen/Gibco), 50% methanol, and 10% acetic acid for 10 min. The staining solution was decanted and the cell monolayer rinsed with distilled water to stop staining of the cells. When necessary, the stained cells were destained to varying degrees with a solution of 50% methanol and 10% acetic acid.

BPT-1 cell growth was assayed at passage 49 by counting the increase in the total cells per T25 flask over a 16 day period at 4-day intervals post-passage. Duplicate T25 flasks were counted at each time interval. Single cell suspensions of the contents of each flask were produced by washing the cells once with 2 ml of 2 M urea. The cells were incubated at ~35°C in the residual urea left behind after aspiration (~0.2 ml) for 5–6 min. One-half milliliter of trypsin–EDTA was added to each T25 flask to finish dissociation of the cells over a further 10 min incubation at ~35°C. The cells were suspended to a total volume of 2 ml in 10% DMEM for cell counts. Total cells per T25 flask was ascertained by averaging the

counts of 16 hemocytometer squares (1 mm^2). Input of BPT-1 cells at the start of the growth assay was unknown but was the result of a 1:30 split ratio from a nearly confluent stock culture. STO feeder cells surviving the urea/trypsin/EDTA dissociation were similarly enumerated from a parallel group of feeder cell T25 flasks that had not received any BPT-1 cell input.

Chromosome Analysis

BPT-1 cells were analyzed for chromosome content at passages 59. BPT-1 cells were harvested to single cells by treatment with 2 M urea and trypsin–EDTA and metaphase spreads were prepared as previously described (Talbot et al., 2000a). Metaphase spreads on replicate slides were stained with 1 μg/ml propidium iodide (PI; Molecular Probes, Eugene, OR) and 2 μg/ml bisbenzimide (Hoechst 33342; Molecular Probes) for fluorescent observation. For chromosome counts, propidium iodide stained metaphase spreads were imaged at ~1,000× magnification using a Zeiss LSM 410 Confocal Microscope equipped with a 63 × C-Apochromat 1.2 NA water immersion objective. For H 3342 fluorescent observation, the 351 nm line of a Coherent Innova 90 laser was used for excitation and the emitted light was passed through a long-pass 397 nm filter. The 568 nm line of an argon/krypton laser was used for excitation of PI and emitted light was filtered through a long pass 590 nm emission filter. Approximately 50 BPT-1 metaphase spreads were counted for chromosome content. Approximately 30 metaphase spreads from a bovine fetal fibroblast cell culture (passage 4) were counted as a comparative control.

Anti-Viral Interferon-tau (IFN-τ) Activity Assay

Anti-viral assays of the conditioned medium (CM) of BPT-1 were completed as described by Roberts et al. (1989). BPT-1 cells were assayed at passages 13 where the 4-day CM was serum-free (SF) DMEM + 1× ITS [insulin (10 μg/ml), transferrin (5.5 μg/ml), and selenium (0.005 μg/ml); Sigma Chemical Co., St. Louis, MO], and at passage 17 where the 4-day CM was 10% DMEM, and at passage 19 where the medium, again 10% DMEM, was conditioned for 3 days. CM from STO feeder cells alone and unconditioned medium were assayed as negative controls. The ability of samples to prevent virus-induced cell lysis by 50% was compared to a recombinant human interferon-alphaA standard (Calbiochem, La Jolla, CA; 3.84×10^8 IU/mg). The concentration of IFN-τ in CM was calculated based on the specific activity of recombinant bovine IFN-τ (rbIFN-τ; $2.52 \pm 0.49 \times 10^8$ IU/mg) included in each assay. Assays were completed in duplicate and results were reported in IU of IFN-τ per ml of culture medium. The assay had a sensitivity of 20 IU per ml.

Immunoblot Analysis of Conditioned Medium

Western blots and immunoprobing of the blots with anti-bovine IFN-τ antibody were completed as described previously (Talbot et al., 2000a) except the proteins were blotted on to PVDF membranes (Millipore, Bedford, MA) and ECL Plus (Amersham Biosciences, Piscataway, NJ) was used for antibody detection. Three milliliters of SF medium (DMEM high glucose) was conditioned for 72 hr by confluent monolayers of BPT-1 trophectoderm at passage 58 (T25 flask) that had been washed 4× with SF medium. The washes were performed to remove traces of serum proteins left behind by the FBS containing growth medium routinely used in the propagation and maintenance of the cell culture. Anti-bIFN-τ antibody, used at a dilution of 1:2,500, was the kind gift of Dr. Michael Roberts (Klemann et al., 1990). Seventy-two hours CM from STO feeder cells alone was assayed as a negative control, and 72 hr CM from passage 92 CT-1 cells (Talbot et al., 2000a) and recombinant bIFN-τ were included as positive controls.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) sample preparation and photomicroscopy were done with the assistance of JFE Enterprises, Brookeville, MD as previously described (Talbot et al., 2000a). Ultrastructural analysis was performed on samples processed from one T25 flask BPT-1 culture that was 10 days post-passage at passage 33.

RESULTS

Establishment of BPT-1 Cell Line

Primary outgrowths of bovine parthenogenetic trophectoderm were initiated from parthenogenotes that had reached the blastocyst stage after 8 days of egg culture. The primary trophectoderm cultures were started by pressing the 8-day-old parthenogenetic blastocysts down onto the feeder cells using 25 gauge hypodermic needles. This punctured the blastocysts and helped the trophectoderm cells adhere to the culture substrate. In the first 24 hr, blastocyst cells appeared to undergo some necrosis, but after several days outgrowths of healthy trophectoderm cells appeared from the central collapsed blastocyst mass and grew out in a radial fashion as a tightly knit epithelial monolayer. Endoderm outgrowths, recognized by their distinct cell and colony morphology, frequently occurred (~20%) in the primary parthenogenetic trophectoderm outgrowths.

Primary parthenogenetic trophectoderm colonies were composed of approximately cuboidal epithelial cells closely packed together in the center of the colony with flatter and more spread out cells at the periphery of the colony. The primary colonies often had occasional domes or areas of the cell monolayer where culture fluid was being transported under the cell sheet by the cells. The trophectoderm cell's cytoplasm was granular in appearance, and many of the cells contained visible lipid droplets. The cells grew predominantly on the plastic substrate and appeared to push the STO feeder cells out of their way as the colony expanded.

After 2 weeks of primary culture, a robust outgrowth that had reached a colony diameter of approximately 1 cm was chosen for secondary culture and was designated bovine parthenogenetic trophectoderm culture

1 (BPT-1). Prior to the first secondary passage, a small patch of endoderm contaminating the primary trophectoderm cell sheet was removed in toto by microdissection with hypodermic needles and aspiration with a micropipette. This was accomplished with 100% efficiency because the endoderm cells were strongly connected to one another so as to form a continuous unit of cells, and because the endoderm was not adherent to the trophectoderm. After several more days of primary culture, the cells were passaged by chopping the primary trophectoderm colony into small pieces using two hypodermic needles and transferring the resulting clumps of BPT-1 cells into a T25 flask containing a STO feeder cell layer. Clumps of BPT-1 cells that attached in the first 24 hr grew into ~1 cm diameter colonies over 2–3 weeks of culture. The culture, henceforth, was passaged by physical disruption of the BPT-1 monolayer (as previously described in Talbot et al., 2000a) every 2–3 week at a 1:3 split ratio onto fresh STO feeder layers. The BPT-1 cell culture grew relatively slowly during the initial secondary culture period (the first 5–10 passages), but, after this 'establishment period,' grew more quickly and was routinely passaged at 1:20 or 1:40 split ratios. After establishment, the cell line had a population doubling time of approximately 96 hr (Figs. 1 and 2). Similar to the primary culture, the established BPT-1 cell line formed monolayers composed of approximately cuboidal epithelial cells (Fig. 3) where most of the cells were closely packed together but did have some areas where the cells were flatter and more spread out.

Chromosome Analysis of BPT-1 Cells

Figure 4 shows the distribution of chromosome counts found from the enumeration of 52 BPT-1 metaphase spreads at passage 59. A unimodal distribution was present with less than half of the cells (42%) having a normal complement of 60 chromosomes. A number of cells were found with hypodiploid (i.e., 47–59 chromosomes) or hyperdiploid (i.e., 62–66 chromosomes) chromosome complements (Fig. 4A). Two metaphase spreads contained chromosome numbers between diploidy and tetraploidy and one other was near tetraploid and another was tetraploid (Fig. 4A). Most of the chromosomes were acrocentrics with the exception of several chromosomes that appeared to result from Robertsonian translocations (Robertson, 1916; Eldridge, 1985), i.e., centric fusions (Fig. 5A,B). These abnormal chromosomes presented as metacentric or submetacentric chromosomes, and appeared to be a common feature in the metaphase spreads (Fig. 5A,B). Thus, the apparent dipoid counts present in 42% of the spreads may actually represent a hyperdiploid genetic content. When the X-chromosome could be unequivocally identified (submetacentric with no distinctly PI stained centromeric region), their appeared to be only one copy present (Fig. 5A,B).

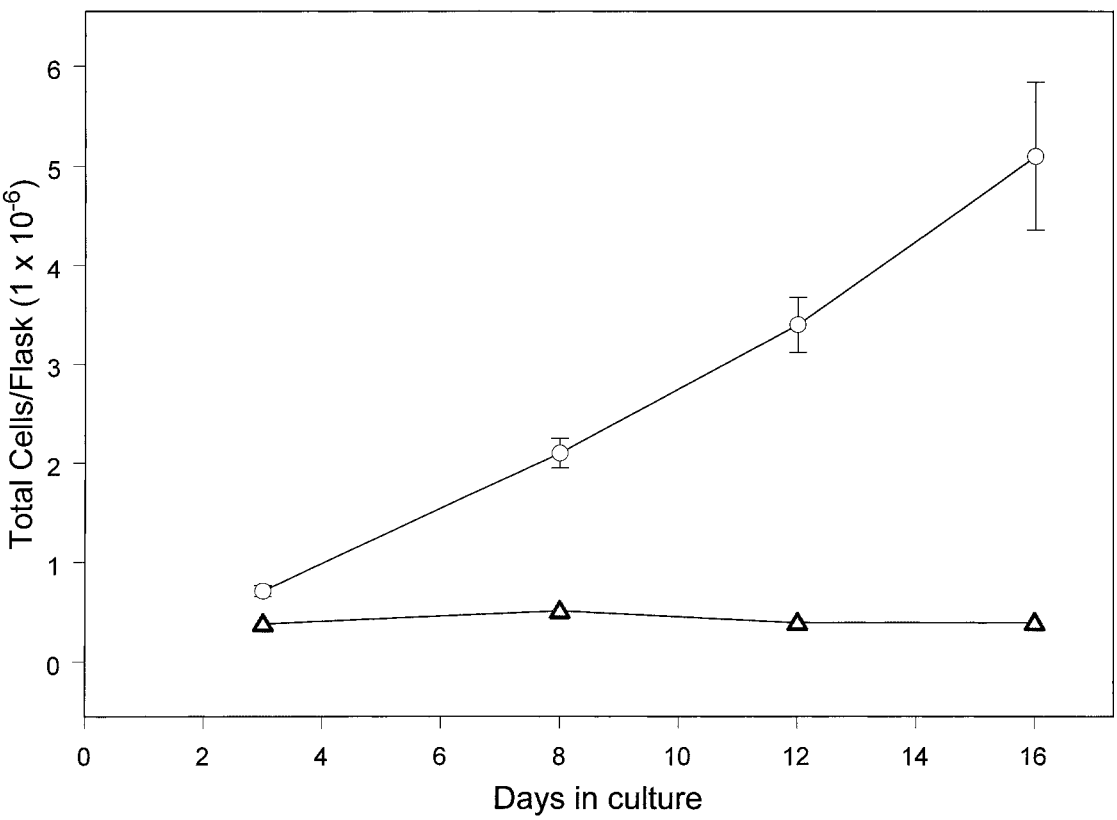

Fig. 1. Growth curve of BPT-1 cells assayed at passage 49. Total of BPT-1 and nondividing STO feeder cells (○). STO feeder cells only (Δ).

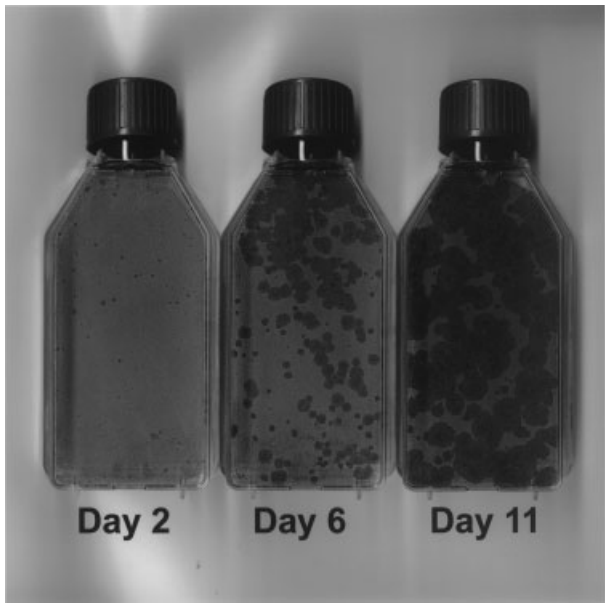

Fig. 2. Growth of BPT-1 cells shown in a cohort of T25 flasks passaged at a 1:20 split ratio at passage 50. Individual flasks were sequentially fixed and stained with Coomassie Blue R-250 over a 11 day post-passage culture period to illustrate the growth of the cells.

As a comparative control, a secondary culture of bovine fetal fibroblasts (BFF) were assayed at passage 4. Of 31 BFF metaphase spreads examined, 74% were diploid containing the full complement of 58 acrocentric chromosomes and two submetacentric X-chromosomes (Figs. 4B and 5C). The remaining BFF metaphase spreads were near diploid and one was tetraploid (Figs. 4B and 5C).

Interferon-tau Expression by BPT-1 Cells

Nearly confluent cultures of BPT-1 were assayed for secreted IFN-τ by anti-viral assay at passages 13, 17, and 19 (Table 1). The two independent CM samples taken at passage 13 were in SF medium conditioned for 4 days. The 4-day CM at passage 17 and 3-day CM at passage 19 were collected in DMEM containing 10% FBS. The result showed the BPT-1 cells were secreting anti-viral activity equivalent to several hundred ng/ml of IFN-τ per ml of culture medium (Table 1). The 3-day CM sample had approximately 10-fold less activity than any of the 4-day samples. The medium conditioned for 4 days by STO feeder cells alone was below the detection limits of the anti-viral assay for interferon activity.

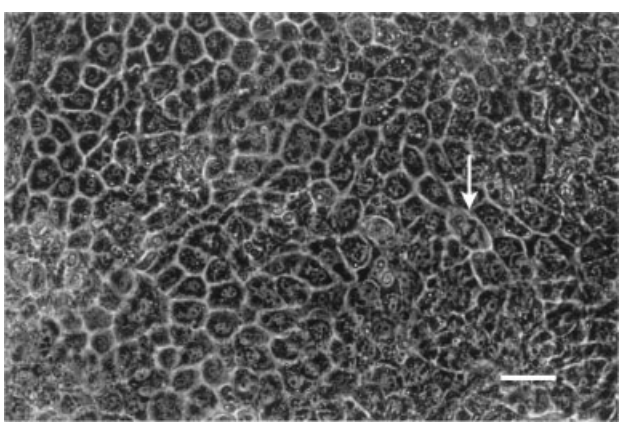

Fig. 3. Phase-contrast light micrograph of BPT-1 cells at passage 61. Arrow indicates a BPT-1 cell in metaphase. Scale bar = 40 μm.

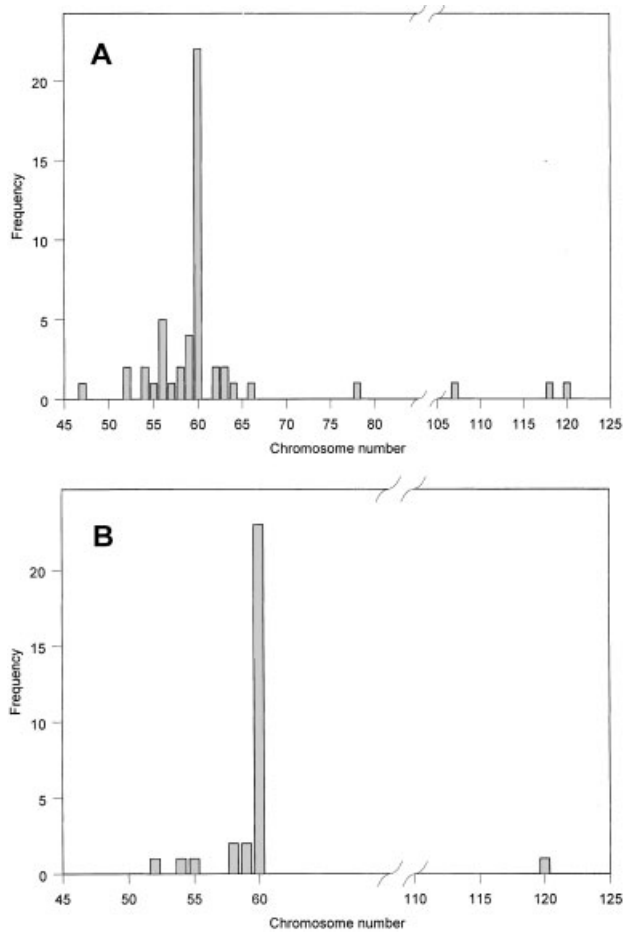

Fig. 4. Chromosome content analysis of BPT-1 cells. **A**: Fifty-two metaphase spreads of BPT-1 cells were prepared and counted at passage 59. **B**: Thirty-one metaphase spreads from a BFF culture at passage 4 were prepared and counted as a comparative control.

A Western blot of samples of SF media conditioned for 72 hr by BPT-1 at passage 58 or STO feeder cells only were analyzed for IFN-τ content by probing with anti-IFN-τ antibody. The purified recombinant IFN-τ positive control was detected by the antibody and produced a band with an apparent molecular weight of 18–19 kDa as would be expected of the nonglycosylated form of the protein (Fig. 6, lane 1; Klemann et al., 1990). Several proteins ranging from 20 to 23 kDa were detected by the antibody in the BPT-1 CM as would be expected for the differentially glycosylated isoforms of IFN-τ (Fig. 6, lane 2). The IVF-derived trophectoderm cell line, CT-1 (passage 92), also secreted IFN-τ into its medium as was previously shown for the CT-1 cells at earlier passage levels (Fig. 6, lane 3; Talbot et al., 2000a). Similarly prepared CM from STO feeder cells alone were negative

for IFN-τ protein (Fig. 6, lane 4). Total protein staining of the PVDF blots with Coomassie Blue-R250 (Fig. 6; lanes 5–9) indicated a close coincidence in molecular weight with purified nonglycosylated rbIFN-τ and, futhermore, that the BPT-1 cells were not secreting proteins typical of serum-protein profiles as is found from bovine visceral endoderm cell cultures (Talbot et al., 2000a).

TABLE 1. Antiviral Activity of Conditioned Culture Medium[a]

Sample[b]	bINF-τ (IU/ml)[c]	bINF-τ (ng/ml medium)
BPT-1 (P13; 4d SFM)	210,118 ± 74,666	833 ± 296
BPT-1 (P13; 4d SFM)	198,930 ± 118,986	788 ± 471
BPT-1 (P17; 4d SCM)	371,431 ± 107,226	1,471 ± 425
BPT-1 (P19; 3d SCM)	12,524 ± 4274	50 ± 17
STO feeder only (4d SFM)	ND	ND

[a]Assay sensitivity was ≤20 IU/ml.
[b]P, number of passages in culture; SFM, serum-free medium; SCM, serum-containing medium.
[c]ND, not detectable.

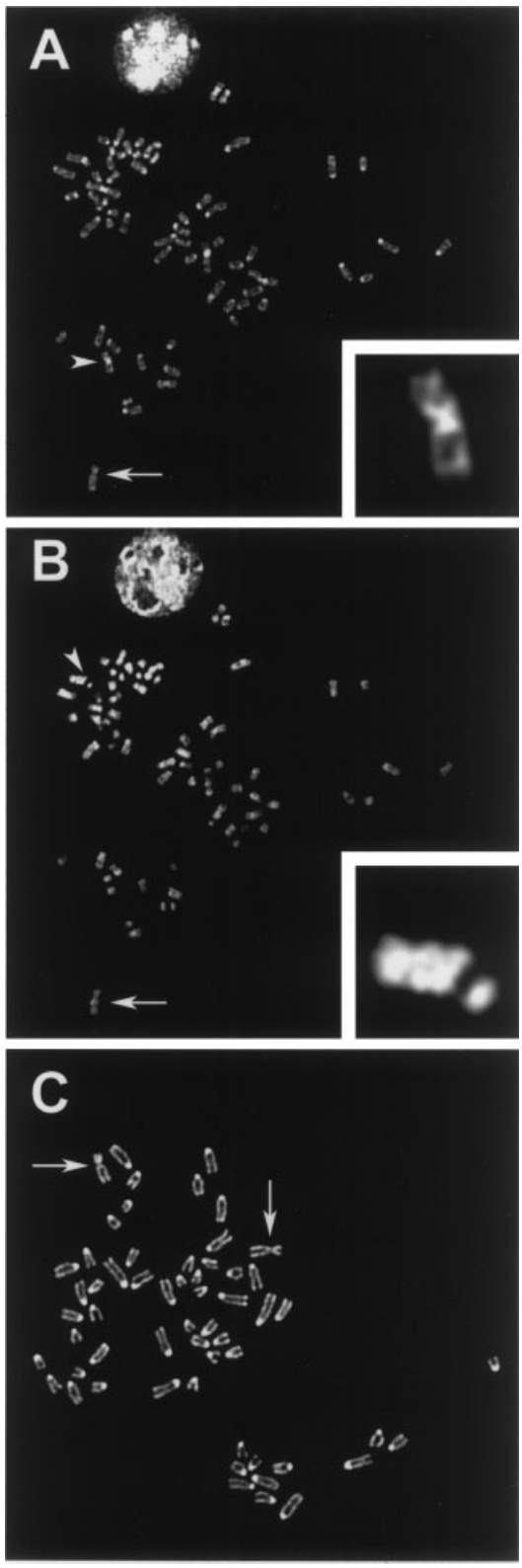

Fig. 5. Karyotype of BPT-1 cells at passage 59. **A**: BPT-1 metaphase spread stained with propidium iodide (PI). Arrow indicates the single X-chromosome present and arrowheads indicate translocated and centromere fusion chromosomes (one translocation event chromosome enlarged in inset). Note the relatively bright staining of centromeres with PI. **B**: Same BPT-1 metaphase spread as in (A) stained with Hoechst 33342; note lack of centromere staining and one translocation event chromosome enlarged in inset. **C**: Passage 4 BFF metaphase spread stained with PI. Arrows indicate X-chromosomes; note lack of centromere staining in the X-chromosomes compared to robust PI staining of the centromeres of the acrocentric autosomes.

Transmission Electron Microscopic (TEM) Analysis of the BPT-1 Cell Line

BPT-1 cells were arranged in a single layer of elongated cuboidal cells growing on top of or between the STO feeder cells (Fig. 7A). While the BPT-1 cells were never intimately joined to the STO feeder cells, they were usually in close proximity to the STO cells, except in the case where dome formation occurred. Numerous strands or bundles of strands of collagen were often interspersed between the STO feeder cells and the BPT-1 cells (Fig. 7A). Prominent and numerous microvilli at the apical surface (facing the medium) marked the polarized morphology of the BPT-1 cells, and the cells were joined by numerous desmosomal elements and tight junctional (TJ) complexes at their lateral surfaces (Figs. 7A and 8). A thin but distinct basement membrane ran along, and in close proximity to, the bottom of the BPT-1 cells (Fig. 9). Golgi complexes, smooth and rough endoplasmic reticulum, mitochondria and microfilaments were all numerous and well represented in the BPT-1 cells, and large and small lipid vacuoles were also commonly found in the cells (Fig. 7A). Overall the BPT-1 cells were similar in ultrastructure presentation to the trophectoderm cells of the CT-1 cell line (Talbot et al., 2000a) and were also remarkably similar to the trophectoderm cells found in an in vivo filamentous stage bovine blastocyst (Fig. 7B).

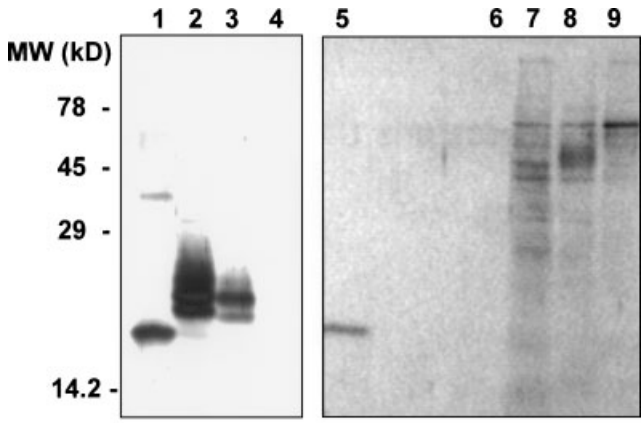

Fig. 6. Immunoblot of BPT-1 conditioned medium (CM) demonstrating specific expression of bIFN-τ. Western blots of 1× CM (72 hr) were probed with anti-bIFN-τ (**lanes 1–4**), or were stained for total protein with Coomassie Blue (**lanes 5–9**). Lane 1 and 6 = 500 pg of rbIFN-τ.; lane 2 and 7 = BPT-1 at passage 58 (15 μl); lane 3 and 8 = CT-1 at passage 92 (15 μl); lane 4 and 9 = STO feeder cell alone (15 μl), and lane 5 = 100 ng rbIFN-τ. Intervening wells between lanes 5 and 6 were blank.

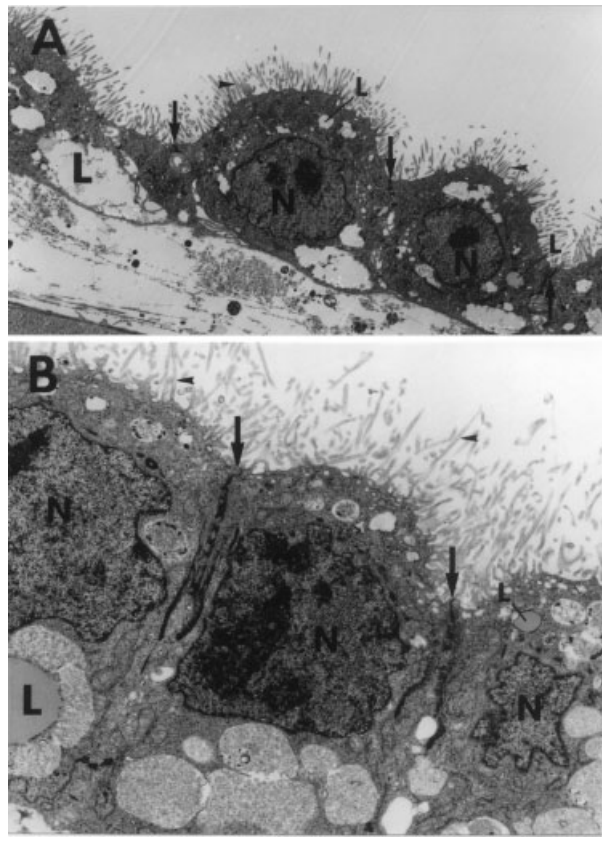

Fig. 7. Transmission electron micrograph of BPT-1 cells in comparison to the trophectoderm cells of a 19-day in vivo bovine blastocyst. **A**: Passage 33 BPT-1 monolayer culture grown on STO feeder cells (feeder cells, not visible, are underneath the BPT-1 cells). ×4,800. **B**: In vivo trophectoderm cells from near the tip end of a 19-day filamentous stage bovine blastocyst (endoderm cells, not visible, are situated underneath the trophectoderm layer). Note the relatively long microvilli (arrowheads) and tight junctional (TJ) connections (arrows) at the apical (facing the uterine environment) portion of the cells. Desmosomal connections below the TJ are particularly numerous and pronounced in the in vivo cells, but are also prominent between the BPT-1 cells. ×9,450. N, nucleus; L, lipid vacuoles.

DISCUSSION

The study demonstrates that a parthenogenetic bovine trophectoderm cell culture could be established in long term culture. This might not have been assumed since a striking feature of mouse gynogenotes/parthenogenotes development in vivo is poor development of and degeneration of the extraembryonic membranes (Surani et al., 1986; Tada and Takagi, 1992), much of which is composed of trophoblast lineage cells (Gardner, 1983). However, ruminant extraembryonic tissue development is distinctly different from the mouse, and studies of in vivo development of parthenogenotes in sheep indicated either no extraembryonic tissue abnormalities (Loi et al., 1998) or some hypertrophy and hypervascularization (Hagemann et al., 1998). Also, because cell growth in culture is without the complex interdependent interaction found in vivo, the establishment of continuous cell cultures from uniparental tissue sources is perhaps not surprising (Kaufman et al., 1983; Kharroubi et al., 2001). Since the BPT-1 cells have been continuously cultured for nearly 3 years and for greater than 70 passages, usually at high split ratios, it is probable that the culture is immortalized and is a cell line.

The BPT-1 cell line was near diploid based on the fact that a large percentage (42%) of the metaphase spreads assayed contained the normal complement of 60 chromosomes and that the majority of the remaining spreads showed chromosomes counts between 55 and 65. This level of heteroploidy of BPT-1 is not unexpected for an extensively passaged cell line and chromosomal anomalies such as translocations also typically occur over passage (Freshney, 1994; Mamaeva, 1998). However, the several Robertsonian translocations (Robertson, 1916; Eldridge, 1985) observed probably occurred at the one cell stage because they occur most commonly in the oocyte during meosis (Bandyopadhyay et al., 2002), because parthenogenotes have abnormal spindle formations at first division (Navara et al., 1994), and because of the tendency of unpaired chromosomes to divide abnormally at their centromeres (Zhang et al., 2001). The trophectoderm of the initial cell culture was presumably diploidized early on after parthenogenetic activation, thus giving rise to a normal or near normal karyotype (Hogan et al., 1994). Karyotype studies of parthenogenetic bovine blastocysts produced in a similar manner as here (sequential ionomycin and 6-dimethylaminopurine treatment) have shown that most of the resulting blastocysts contain cells that are not diploid, but are instead polyploid with mixoploidy being in evidence (Winger et al., 1997; De La Fuente and King, 1998). Thus, it is possible that the BPT-1 cell culture was initially of mixed ploidy, but that diploid trophectoderm cells that were present in the primary explant culture thrived and out competed cells of abnormal ploidy. Subsequent drift in the population to a near diploid

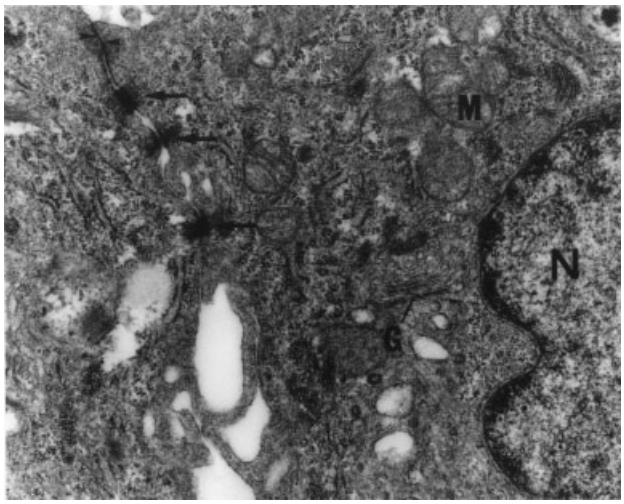

Fig. 8. Transmission electron micrograph of BPT-1 cells. Two BPT-1 cells shown joined by an apical tight junction region (arrowheads) and three lateral desmosomes (arrows). ×60,000. G, Golgi complex; M, mitochondria; N, nucleus.

(slightly hyperdiploid) state probably followed as a result of the BPT-1 cell lines extensive passage in culture as this was previously observed with the IVF-derived CT-1 cell line (Talbot et al., 2000a).

To the extent tested, the BPT-1 cell line was substantially similar to the CT-1 cell line (Talbot et al., 2000a) previously isolated from a blastocyst produced by IVF. Cell morphology by light microscopy and TEM, IFN-τ production, growth rate, colony-morphology, and apparent immortilization in culture were all similar to the CT-1 cell line. Differences between the cell lines based on source material, i.e., IVF blastocyst vs. parthenogenetic blastocyst, should presumably exist. As with any in vitro model, it is hoped that comparative studies of the gene expression or protein expression in the two in vitro models will reveal differences relevant

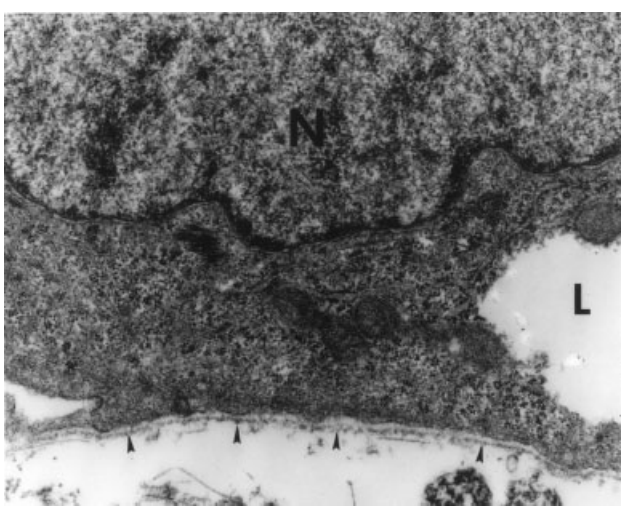

Fig. 9. Transmission electron micrograph of basement membrane underlaying BPT-1 cell. ×60,000. L, lipid vacuole; N, nucleus.

to in vivo development. However, discerning differences resulting from parthenogenesis from differences that are present as a result of cell culture, i.e., environmental and cellular stochastic mechanisms, will require the comparison of numerous cell lines derived from the two sources. For example, at present a notable difference between the two cell lines is that, unlike the CT-1 cells, the BPT-1 cells are difficult to culture in the absence of STO feeder cells (unpublished observation) and that the BPT-1 cells appear to secrete more IFN-τ than the CT-1 cells (Fig. 5). Further evaluation of both of these observations will need to be done in comparison to other independently derived trophectoderm cell lines to determine the relevance and consistency of these phenotypes.

The BPT-1 parthenogenetic cell line, and similar cell lines that could be created in future, may help define deficiencies in "reprogramming" that lead to placentation abnormalities in NT pregnancies (Hill et al., 2000; De Sousa et al., 2001; Hashizume et al., 2002). In comparison with NT embryos and the successful reprogramming of the donor somatic nucleus, IVF-derived trophectoderm represents the best case comparative developmental potential (often successful) and the parthenogenote-derived trophectoderm the worst case comparative developmental potential (never successful). Thus, analysis of trophectoderm cell lines derived from IVF embryos (i.e., bovine embryos carrying a normal maternal and paternal genetic complement) or parthenogenotes (maternal genetic complement only) should allow the definition of many genetic and phenotypic differences that predispose parthenogenetic embryos to placentation failure and early death (Surani and Barton, 1983; Fukui et al., 1992; Boediono and Suzuki, 1994; Susko-Parrish et al., 1994; Loi et al., 1998). That in vitro models will yield reliable gene expression profiles is supported by the finding that in fibroblast cultures derived from uniparental fetuses (androgenotes or parthenogenotes), the parent-of-origin allele-specific expression profile of several maternally imprinted and several paternally imprinted genes were maintained over 30 cell generations in culture (Kharroubi et al., 2001). Aberrant alterations in gene expression arising from the lack of normal imprinted gene compliments in parthenogeneitc trophectoderm (Surani and Barton, 1983; Surani et al., 1990) may provide mechanistic gene expression candidates for placentation failure that can be similarly found in NT trophectoderm gene expression, but which, in the case of NT, result from ineffective reestablishment of genetic imprint status or methylation status (Humpherys et al., 2002; Cezar et al., 2003; Santos et al., 2003). The recent report that certain DNA methylation anomalies were localized to the trophectoderm cells of bovine NT blastocysts also highlights that the analysis of in vitro models of trophectoderm tissue might be useful in determining epigenetic deficiencies that lead to placental dysfunction (Kang et al., 2002).

In conclusion, in vitro models offer ready opportunities for manipulation of the cell environment. Thus, besides the "standard" culture conditions, the parthe-

nogenetic trophectoderm cells can be experimentally tested with the addition or subtraction of specific growth factors, hormones, metabolites, etc., to ask how gene expression responses are changed in themselves and in comparison to IVF- and NT-derived trophectoderm cell cultures. Further, it is possible that modeling of trophectoderm differentiation can also be studied. For example, a specific culture substrate condition has recently been shown to stimulate trophectoderm binucleate cell differentiation in the BT-1 bovine trophectoderm cell line, and expression of placental lactogen by the in vitro-produced binucleated cells was demonstrated (Nakano et al., 2002). Thus, an in vitro model of the cellular events that occur during placentation may eventually be possible.

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